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Telomerase Activity in Esophageal Carcinoma

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Background and Objectives: Telomerase is a ribonucleoprotein that synthesizes telomeric DNA. Immortalized and carcinoma cells show no loss of telomere length during cell division. Telomerase activity has been demonstrated in carcinomas of various organs, but not in nonneoplastic tissues. In patients with esophageal carcinoma, no data have been reported concerning the relationship between telomerase activity and clinicopathological findings.

Materials and Methods: Esophageal carcinomas from 31 patients and normal esophageal mucosae from 92 patients were examined. Telomeric Repeat Amplification Protocol assay to detect telomerase activity and Southern blot analysis to examine telomere length were performed.

Results: Of the 31 carcinomas, 27 (87%) had detectable telomerase activity. Twenty-one (23%) of the 92 normal esophageal mucosae from autopsied patients also had detectable telomerase activity. There was no difference between stage and outcome and absence or presence of telomerase activity. No difference in terminal restriction fragment (TRF) length was observed between carcinomas with and without telomerase activity.

Conclusion: Telomerase activity was demonstrated in a considerable number of normal esophageal mucosae. This suggests the possibility of a high frequency of false positivity if the presence of telomerase activity alone is used as a tumor-specific marker.

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KEY WORDS: **esophagus; telomere; squamous cell carcinoma; small cell carcinoma; adenosquamous carcinoma; adenocarcinoma**

INTRODUCTION

Telomerase is a ribonucleoprotein that synthesizes telomeric DNA located at chromosomal ends using a segment of RNA within its molecule as a template [1,2]. Telomeres are composed of many hundreds of tandem repeats of the sequence TTAGGG and play a role in chromosomal protection and replication [3]. In one normal somatic cell division, chromosomes lose 50–200 base pairs of the telomeric sequence [4,5], and thus telomeres shorten progressively with age. In contrast, immortalized and carcinoma cells show no loss of telomere length during cell division [6]. Telomerase activity has been demonstrated in carcinomatous tissues of various organs, but not in normal somatic cell lines or nonneoplastic tissues adjacent to the carcinomas [7]. These facts suggest that telomerase activity is directly involved

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in protection against telomere shortening and cell death [7].

Up to now, however, no data have been reported concerning the relationship between telomerase activity and clinicopathological findings in patients with esophageal carcinoma. In the present report, we detail telomerase activity in esophageal carcinomas and describe various findings including patient outcome and histopathological features.

MATERIALS AND METHODS

Tissues

Resected esophageal carcinomas from 31 consecutive patients (male: 28, female: 3, mean age: 62 years) treated surgically at Saitama Cancer Center Hospital between May 1989 and February 1991 were examined. Specimens of normal esophageal mucosa obtained from 92 autopsy patients (mean age: 81 years) at Tokyo Metropolitan Geriatric Hospital were also used. None of the 92 patients had carcinomas in the esophagus or other organs.

All the samples of esophageal carcinoma and normal esophageal mucosa were stored at −80°C until use.

Telomeric Repeat Amplification Protocol (TRAP) Assay [7]

Lysates were prepared by powdering tissues frozen under liquid nitrogen, followed by homogenization in 200 μl of ice-cold lysis buffer [10 mM Tris–HCl (pH 7.5)–1 mM $MgCl_2$–1 mM EGTA–0.1 mM phenylmethylsulfonyl fluoride–5 mM β-mercaptoethanol–0.5% CHAPS–10% glycerol] and incubation for 30 min on ice. After the incubation, the lysates were centrifuged at 10,000 × g for 20 min at 4°C and the supernatant and precipitate were rapidly frozen separately and stored at −80°C. Protein concentration of the supernatant was determined by the Bradford assay (Bio-Rad, Hercules, CA). Assay tubes were prepared by sequestering 0.1 μg of CX primer (5′-CCCTTACCCTTACCCTTACCTAA-3′) under a wax barrier (Ampliwax; Perkin-Elmer Cetus, Foster City, CA). The extracts, equivalent to 6 μg protein, were assayed in 50 μl of reaction mixture containing 20 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, 63 mM KCl, 0.005% Tween 20, 1 mM EGTA, 50 μM dNTPs, 150 kBq [α-^{32}P] dCTP, 0.1 μg of TS primer (5′-AATCCGTCGAGCAGAGTT-3′) (United States Biochemicals, Cleveland, OH), 1 μg T4 gene 32 protein (Boehringer Mannheim, Germany) and two units of Taq DNA polymerase (GIBCO-BRL, Gaithersburg, MD). After a 30-min incubation at room temperature for telomerase-mediated extension of the TS primer, the reaction mixture was heated at 90°C for 90 sec and then subjected to 31 polymerase chain reaction (PCR) cycles at 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec. The product was then electrophoresed on a 10% polyacrylamide gel.

Southern Blot Analysis

Genomic DNA was isolated from the frozen precipitate by incubation with 400 μl of lysis buffer (10 mM Tris–HCl (pH 7.4), 50 mM EDTA, 50 mM NaCl, 27% sucrose) containing 0.3 mg of proteinase K (Merck, Rahway, NJ) and 0.8% SDS at 37°C overnight. Then, the DNA was extracted twice with phenol and chloroform and precipitated with isopropanol. The DNA precipitate was dissolved in TE (10 mM Tris HCl, 1 mM EDTA, pH 8.0). Ten micrograms of the DNA was digested with Hinf1, and 7 μg of the digested DNA was electrophoresed on 0.8% agarose gels and transferred to nylon membranes (Hybond-N+, Amersham, UK). The membranes were prehybridized at 65°C in hybridization solution containing 6×SSPE, 1% SDS, and 50 μg/ml salmon sperm DNA, then hybridized overnight at 50°C with a ^{32}P-end-labelled $(TTAGGG)_4$ telomeric probe. The filters were washed once in 2×SSC at room temperature, twice in 6×SSC–0.1% SDS at 50°C, and then autoradiographed. The length of the terminal restriction fragment (TRF) was estimated at the peak of the hybridization signal. Differences in TRF length among patients in their 50s, 60s and 70s were tested by the Kruskal-Wallis test. Other statistical analyses were performed using the Student's *t* test.

Clinicopathological Observation

All esophageal specimens from surgical and autopsy cases were examined histopathologically. The tumors were classified into histological types, mainly according to the WHO tumor classification [8] and staged according to the TNM classification of the UICC [9]. Survival curves were computed actuarially using the Kaplan-Meier method. The significance of related survival between the two patients groups, with or without telomerase activity, was tested using the Cox-Mantel method.

RESULTS

Histologically, the 31 carcinomas comprised 25 squamous cell carcinomas, two undifferentiated small cell carcinomas, two adenosquamous carcinomas, one adenocarcinoma, and one basaloid squamous carcinoma.

Twenty-seven (87%) of the 31 carcinomas had detectable telomerase activity (Table I). The activity was present in 22 (88%) of the 25 squamous cell carcinomas, both of the two small cell carcinomas, both of the two adenosquamous carcinomas, and the one adenocarcinoma. Twenty-one (23%) of the 92 normal esophageal mucosae from the autopsy cases also had detectable telomerase activity (Fig. 1). Four tumors without telomerase activity were at stages 2A to 4, and the re-

TABLE I. Clinicopathology, Telomerase Activity, and Telomere Length in Carcinomas From 31 Patients With Esophageal Carcinoma

No.	Age	Sex[a]	Stage	Histology[b]	Telomerase activity[c]	TRF[d] (kbp)	Survival time[e]
1	46	m	4	SCC	+	7.5	8+
2	51	m	2A	SCC	+	13.0	69−
3	51	m	3	SCC	+	5.0	5+
4	52	m	1	SCC	+	7.0	63−
5	54	m	3	SCC	+	22.0	22+
6	57	m	3	SCC	+	3.5	8+
7	57	m	2B	SCC	+	5.5	23+
8	58	m	3	SCC	+	23.0	9+
9	61	m	3	SCC	+	7.0	14+
10	63	m	3	SCC	+	3.5	9+
11	63	m	4	SCC	+	3.0	62−
12	64	m	3	SCC	+	3.2	10+
13	65	m	3	SCC	+	6.0	36+
14	65	m	3	SCC	+	9.4	47−
15	66	m	3	SCC	+	18.0	4+
16	66	m	3	SCC	+	8.0	10+
17	70	m	3	SCC	+	9.4	21+
18	74	m	2A	SCC	+	5.3	50+
19	75	m	3	SCC	+	6.3	13+
20	77	m	2A	SCC	+	5.5	18+
21	77	m	3	SCC	+	7.2	6+
22	79	f	2A	SCC	+	4.8	6+
23	60	m	4	UD	+	9.0	8+
24	65	f	3	UD	+	2.3	6+
25	58	m	3	AD	+	6.0	87−
26	60	m	3	AS	+	9.5	39+
27	72	m	3	AS	+	3.0	0+
28	57	m	3	SCC	−	8.5	86−
29	60	m	2A	SCC	−	6.2	75+
30	72	f	3	SCC	−	8.0	2+
31	53	m	4	BCC	−	6.5	22+

[a]m: male; f: female.
[b]SCC: squamous cell carcinoma, UD: undifferentiated small cell carcinoma, AD: adenocarcinoma, AS: adenosquamous carcinoma, BCC: basaloid squamous carcinoma.
[c]+: positive telomerase activity, −: negative telomerase activity.
[d]TRF: terminal restriction fragments.
[e]survival time (months), +: dead, −: alive.

maining 27 tumors with telomerase activity were at stages 1–4.

Among the seven cases at stages 1 to 2B, six (86%) carcinomas had telomerase activity, and 21 (88%) of the 24 tumors at stages 3–4 had telomerase activity. No relationship between telomerase activity and patient survival time was observed (not shown).

TRF lengths in each carcinoma are shown in Table I. The TRF lengths for esophageal carcinomas with telomerase activity ranged from 2.3 to 23.0 kilobase pairs (kbp), whereas those for carcinomas without the activity ranged from 6.2 to 8.5 kbp. The mean TRF length for tumors with telomerase activity was 7.9 kbp and that for tumors without the activity was 7.3 kbp. The mean TRF lengths for esophageal carcinoma cells were 10.0 kbp for 10 patients in their 50s, 7.1 kbp for 12 in their 60s, and 6.2 kbp for 8 in their 70s. Although shortening of the mean TRF length was thus noted in older patients, no significant differences among the three age groups were revealed by the Kruskal-Wallis test. The TRF length for normal esophageal mucosae ranged from 9.0 to 16.0 kbp, with a mean of 12.7 kbp. The *t* test showed that the TRF length for normal esophageal mucosa was significantly greater than that of tumor cells ($P = 0.01$).

DISCUSSION

Telomerase activity has been reported to be recognizable in carcinomas originating from many different organs [7]. Here, we have added further data indicating that esophageal carcinomas also have a high incidence of detectable telomerase activity. Histologically, most esophageal carcinomas in Japan are squamous cell carcinomas. In this study, two undifferentiated small cell

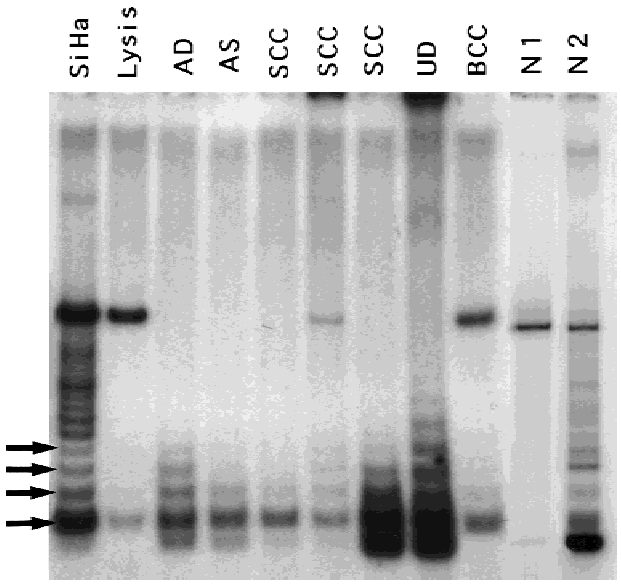

Fig. 1. Telomerase activity in control and esophageal carcinoma. The telomerase activity was detected in control SiHa cells and all the histological types of esophageal carcinoma, except for BCC, and was not detected in the control lysate. The activity was not detected in normal esophageal mucosa (N1), but was detected in another specimen of normal mucosa (N2). SiHa: positive control for SiHa cells, Lysis: negative control for lysis buffer, AD: adenocarcinoma, AS: adenosquamous carcinoma, SCC: squamous cell carcinoma, UD: undifferentiated small cell carcinoma, BCC: basaloid squamous carcinoma, N1 and N2: normal mucosae of autopsy patients. Arrows: 6-bp telomerase ladder signals in control SiHa cells.

carcinomas, two adenosquamous carcinomas, and one adenocarcinoma of the esophagus also were found to have detectable telomerase activity.

With regard to stomach carcinoma, it has been reported that patients with telomerase-positive tumors show a poorer prognosis than those with telomerase-negative tumors [10]. Although the negative group in this study was small, we were unable to demonstrate any clear relationship between the absence or presence of telomerase activity and tumor stage or patient survival time. Moreover, telomerase activity was demonstrated in early-stage as well as late-stage esophageal carcinomas. However, telomerase activity is considered to be absent in normal tissues except for ovary, testis, and hematopoietic stem cells. Yet, in one study, intestinal metaplastic gastric mucosa was reported to have the activity in 2 (15%) of 13 samples (10). In this study, despite the use of autopsy samples, 23% of normal mucosae from patients without malignant diseases also had detectable telomerase activity. Kim et al. [7] also reported that some noncancerous tissues from the head and neck, kidney, and breast adjacent to carcinomas also had telomerase activity. In the small and large intestines, migration of columnar cells from the proliferative zone to the intestinal lumen requires 2–3 days [11]. In the esophagus, the proliferative zone is a basal layer adjacent to the basement membrane. In mice, replacement of the esophageal mucosal epithelium takes 7 days [12]. In any event, the intestinal mucosa and esophageal mucosa show rapid renewal. Previous data including our results suggest that sites of rapid renewal in mucosal epithelia, i.e., digestive tract mucosa, have detectable telomerase activity, like ovarian and testicular tissues. In addition, in this study it is possible that telomerase activity may have been detected in infiltrating lymphocytes, reported to possess the activity, in the esophageal mucosa. Nonneoplastic peripheral lymphocytes have been reported to have telomerase activity [13] and the esophageal epithelium normally contains lymphocytes [14]. Furthermore, the lamina propria also contains lymphocytes with well-developed lymph follicles occasionally [15].

To date, there have been no reports indicating whether biopsy specimens of the normal esophageal mucosa have telomerase activity. Although this activity seems to be one of the most important tumor specific markers reported to date and can be detected easily in the esophagus, a considerable number of noncancerous esophageal mucosae had detectable telomerase activity in this study. This would seem to suggest a high frequency of false positivity if the presence of telomerase activity alone is used as a tumor-specific marker.

In this study, we examined the length of the TRF as an indicator of telomere length. The mean TRF lengths for tumors with and without telomerase activity were 7.9 and 7.3 kbp, respectively, and no relationship between telomerase activity and TRF length could be demonstrated. However, carcinomas with telomerase activity showed a greater variety of TRF length than those without the activity. Our statistical analysis failed to demonstrate any significant differences in tumor TRF length among three age groups.

The TRF length for esophageal carcinoma was significantly less than that of normal mucosa, suggesting that the telomere shortens during carcinogenesis in the esophagus, or that esophageal carcinoma arises from mucosal cells having shortened telomeres.

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